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Further evidence for the existence of NK_2 tachykinin receptor subtypes

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1 We have evaluated the biological activity of a number of neurokinin A (4–10), (NKA (4–10)) analogues in the endothelium-deprived rabbit isolated pulmonary artery (RPA) and hamster isolated trachea (HT), two tissues rich in different NK_2 receptor subtypes.

2 MDL 28,564, a pseudopeptide selective for NK_2 receptor sites, behaved as a full agonist in the RPA, while in the HT it competitively antagonized NKA or [βAla^8]-NKA (4–10) contractile effects.

3 The peculiar behaviour of MDL 28,564 in the RPA and HT may be explained neither by a difference in receptor reserve between the two organs (the reserve being three times greater in RPA than in the HT) nor by a different affinity for the two receptor subtypes (identical dissociation constants, pK_A or pK_B, calculated in the RPA and in the HT). On the other hand, MDL 28,564 displayed a very different intrinsic efficacy for the two receptor subtypes.

4 The novel peptides MEN 10,295 ([Trp^7, βAla^8]-NKA-(4–10)) and MEN 10,296 ([Tyr^5, Trp^7, βAla^8]-NKA-(4–10)) behaved as weaker agonists than MDL 28,564 in the RPA, but retained appreciable agonist activity also in the HT.

5 The novel peptides: MEN 10,282 ([Tyr^5, D-Trp^{6,8}, Trp^9, Arg^{10}]-NKA-(4–10)), MEN 10,449 ([diI-Try^5, D-Trp^{6,8,9}, Arg^{10}]-NKA-(4–10)) and the cyclic hexapeptide L 659,877 (cyclo [Leu-Met-Gln-Trp-Phe-Gly]) behaved as competitive antagonists against NKA contractile effects both in the RPA and HT. MEN 10,282 and MEN 10,449 were unable to distinguish between the NK_2 receptor subtypes, having almost the same affinity in the two organs. On the other hand L 659,877 was about 15 times more potent in the HT than in the RPA.

6 These results provide further evidence for NK_2 receptors heterogeneity and are useful in outlining pharmacological features of the two subtypes present in the RPA and HT.

Keywords: Tachykinins; tachykinin receptors; NK_2 receptor subtypes; tachykinin antagonists; pseudopeptides

Introduction

The tachykinin (TK) receptors (NK_1, NK_2, NK_3) have been defined on the basis of their sensitivity to the natural peptides, substance P (SP), neurokinin A (NKA) and neurokinin B (NKB) and to a number of synthetic TK agonists selective for one of the three TK receptors (Buck *et al.*, 1984; 1988; Lee *et al.*, 1986; Drapeau *et al.*, 1987; Dion *et al.*, 1987; Regoli *et al.*, 1987; 1988; 1989; Maggi *et al.*, 1987; Rovero *et al.*, 1989). The identification, through cloning studies of three receptor proteins belonging to the G-protein superfamily, which possess the pharmacological character of the above receptors, supports this classification (Masu *et al.*, 1987; Yokota *et al.*, 1989; Hershey & Krause, 1990).

However, a number of experimental observations obtained with novel TK antagonists are not explained by the present classification of TK receptors. For example, McKnight *et al.* (1988) found that L 659,877 and other cyclic TK antagonists possess a very different affinity (pA_2 values) when tested in two isolated organs endowed with NK_2 receptors, the rat vas deferens and guinea-pig trachea, suggesting the existence of a distinct receptor in the latter organ (termed NK_4). Further, Van Giersbergen *et al.* (1991) have reported that L 659,877 is about 20 times more potent in inhibiting [^{125}I]-NKA specific binding on NK_2 receptor sites present on hamster urinary bladder membranes than on NK_2 sites expressed in a murine fibroblast cell line (SKLKB82#3) transfected with the bovine stomach cDNA.

Recently we found (Maggi *et al.*, 1990) marked differences in the affinity estimates for selective NK_2 antagonists in two preparations which have been shown to contain an apparently homogeneous class of NK_2 receptors: the endothelium-deprived rabbit isolated pulmonary artery (RPA) and hamster isolated trachea (HT). Thus, MEN 10,207 (referred to as peptide I in Maggi *et al.*, 1990), a selective NK_2 antagonist (Rovero *et al.*, 1990), was about 100 times more potent in the RPA than in the HT while a converse picture was obtained with a linear peptide antagonist termed R396 (referred to as peptide III in Maggi *et al.*, 1990). MEN 10,207 exhibits also a marked difference in inhibiting [^{125}I]-NKA binding to NK_2 sites, being about 120 times more potent at the NK_2 receptors expressed by a murine fibroblast cell line (SKLKB82#3), than at the receptors present on hamster urinary bladder membranes (Van Giersbergen *et al.*, 1991).

Collectively, these data suggest that the NK_2 receptor is heterogeneous in respect to antagonists which detect differences in the NK_2 receptor present in different tissues. Here we present further evidence supporting this conclusion. As probes of the NK_2 receptor we have used a number of NK_2 ligands, including L 659,877 and MDL 28,564. This latter compound, a pseudopeptide analogue of NKA-(4–10), maintains selective affinity for NK_2 receptor sites (Harbeson *et al.*, 1990), but exhibits diverging actions in tissues endowed with NK_2 receptors: in the guinea-pig trachea it is a full agonist, while in the hamster urinary bladder or the rat vas deferens it is virtually inactive as an agonist and also possesses antagonist activity (Buck *et al.*, 1990). Because of the peculiar behaviour of MDL 28,564, the 'receptor reserve' for NKA in the RPA and HT has been evaluated, in order to evaluate the role of tissue factors in producing the biological response to TK agonists in the two organs. The affinity constant (K_A) and relative intrinsic efficacy of NKA and MDL 28,564 have also been estimated for TK receptors of RPA and HT.

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Methods

General

Male albino rabbits (3.0–3.5 kg) and Syrian golden hamsters (100–120 g) were stunned and bled. Endothelium-denuded strips of rabbit pulmonary artery (RPA) or rings of the hamster trachea (HT) were excised and prepared for isometric tension recording in oxygenated (96% O_2 and 4% CO_2) Krebs solution in 5 ml organ baths, as described previously (Maggi *et al.*, 1990). Cumulative concentration-response curves to the agonists were obtained, each concentration being added when the effect of the preceding one had reached a steady state. Preliminary experiments indicated lack of desensitization of both RPA or HT to the cumulative administration of peptides. pD_2 values were calculated as $-\log$ molar concentration of agonist producing 50% of maximal effect (EC_{50}). The effect of the TK-antagonists (contact time 15 min) was studied as described previously (Maggi *et al.*, 1990). Antagonist activity was evaluated by the Schild plot method (Arunlakshana & Schild, 1959). Since all TK-antagonists studied yielded a slope not different from unity, the pA_2 values reported were calculated by the constrained plot method (slope constrained to -1) as described by Tallarida *et al.* (1979). Neither antagonists of muscarinic receptors, adrenoceptors and histamine receptors, nor inhibitors of cyclo-oxygenase, lipo-oxygenase or peptidases were used in this study since previous investigations have demonstrated that the tachykinin contractile effect in the RPA and HT is a direct phenomenon, not influenced by the above agents (D'Orleans-Juste *et al.*, 1985; Maggi *et al.*, 1989; 1990). The potency of agonists relative to that of NKA was evaluated by the ratio of the respective EC_{50}s.

The amino acid sequence of peptides used in this study is shown in Table 1.

Determination of dissociation constant (K_A) and receptor reserve values

To determine the agonist affinity (dissociation constant, K_A) and the receptor reserve for NKA in the two bioassays we used the 'partial irreversible blockade' method described by Furchgott (Furchgott, 1966; Furchgott & Bursztyn, 1967). Cumulative concentration-response curves to NKA were constructed both in the RPA and HT, before and after incubation of the tissues with the alkylating agent phenoxybenzamine (Pbz). Previously Pbz, in concentration up to 100 μM, has been shown to reduce NK_2 receptor number without affecting the affinity of iodinated NKA in binding experiments conducted on hamster and rat urinary bladder membranes (Buck & Burcher, 1987). Therefore, to avoid changes in ligand affinity, Pbz concentrations employed in the present study were in the 10–50 μM range, with an exposure time (30 min) which was half of that used by Buck & Burcher (1987). Notably, other investigators working on peptide receptors (Lin & Musacchio, 1983; Vaught *et al.*, 1986) have used alkylating conditions similar to ours.

Pbz was left in contact for 30 min with the tissues. At the end of the incubation period Pbz was washed out repeatedly for 30 min, before repetition of the curve to NKA (40 min after Pbz washout). Concentration-response curves to NKA (or to other TK-agonists), performed in matched preparations before and after incubation for 30 min with the vehicle employed to dissolve Pbz according to the above schedule, proved to be very reproducible, confirming the reliability of the rightward shifts observed in the Pbz-treated tissues. Reciprocal equieffective doses of NKA before and after Pbz treatment were plotted. From the slope and intercept of the straight line fitting the points, the value for K_A and the fraction of receptors remaining unblocked (q) were calculated (Furchgott, 1966; Furchgott & Bursztyn, 1967). Since the extent of alkylation by a given concentation of Pbz was variable, we selected the experiments in which receptors alkylated were at least 50% of total, to minimize the experimental errors produced by low doses of agonist in constructing the reciprocal plot, as suggested by Kenakin (1987). For the same reason, equiactive concentrations of NKA were selected from the upper region of the depressed dose-response curve. The K_A value for MDL 28,564 in the RPA was evaluated in the same manner. Receptor reserve, regarded as the fraction of the total receptor pool not required for a maximal tissue response, is difficult to quantify experimentally. We choose to express the 'reserve' as the ratio between the concentration of NKA required for half maximal receptor occupancy (K_A) and half maximal response (EC_{50}) (Kenakin, 1987). The fractional occupancy of receptors (ρ) required to elicit half maximal response was calculated on the basis of Clark's equation:

$$\rho = \frac{[AR]}{[Rt]} = \frac{[A]}{[A] + K_A} \quad (1)$$

by substituting [A] with EC_{50} and using K_A obtained in each experiment.

Measurement of relative efficacy of neurokinin A and MDL 28, 564

The relative efficacy of MDL 28,564 and NKA ($\varepsilon_{NKA}/\varepsilon_{MDL}$) for the NK_2 receptor in the RPA was calculated by the method of Furchgott & Bursztyn (1967). By this method the relative efficacy of agonists can be obtained from the ratio of their fractional receptor occupancy (ρ) according to:

$$\varepsilon_1/\varepsilon_2 = \rho_2/\rho_1 \quad (2)$$

We calculated 'ρ' by the law of mass action (1), where [A] is the molar concentration of agonist, and K_A is the dissociation constant determined for NKA and MDL 28,564 on paired strips from the same animal by the method of irreversible alkylation with Pbz (see the above section). Control pre-Pbz response data for each agonist were then replotted to obtain response vs log ρ. The antilog of the distance between the two curves along the abscissae, was taken as the relative efficacy (Furchgott & Bursztyn, 1967).

Statistical analysis

Each value in the text, tables or figures is mean ± s.e.mean. Statistical analysis was performed by means of Student's t test for paired or unpaired data. Regression analysis of log concentration-effect curves was performed by the least squares

Table 1 Amino acid sequences of peptides used in this study

NKA-(4–10)	H-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2
[βAla^8]-NKA-(4–10)	H-Asp-Ser-Phe-Val-βAla-Leu-Met-NH_2
MEN 10,295	H-Asp-Ser-Phe-Trp-βAla-Leu-Met-NH_2
MEN 10,296	H-Asp-Tyr-Phe-Trp-βAla-Leu-Met-NH_2
MEN 10,207	H-Asp-Tyr-D-Trp-Val-D-Trp-D-Trp-Arg-NH_2
MEN 10,282	H-Asp-Tyr-D-Trp-Val-D-Trp-Trp-Arg-NH_2
MEN 10,449	H-Asp-(diI)Tyr-D-Trp-Val-D-Trp-D-Trp-Arg-NH_2
MDL 28,564	H-Asp-Ser-Phe-Val-Gly-LeuΨ(CH_2NH)Leu-NH_2
L 659,877	cyclo(Leu-Met-Gln-Trp-Phe-Gly)
R 396	AcLeu-Asp-Gln-Trp-Phe-Gly-NH_2

method, considering linear such curves between 20 and 80% of the maximal response. EC_{50} and 95% confidence limits were calculated accordingly.

Peptides

NKA, NKA (4–10), [βAla^8]-NKA (4–10), MEN 10,295, MEN 10,296, MEN 10,207, MEN 10,282 and MEN 10,449 were synthesized in our laboratory by conventional solid-phase methods. L 659,877 was obtained from Cambridge R.B. (Cambridge, U.K.). MDL 28,564 and R 396 were kind gifts of Dr S.H. Buck, Marion Merrell Dow Research Institute, and Prof. D. Regoli, Department of Physiology and Pharmacology, University of Sherbrooke, Canada, respectively. Phenoxybenzamine (Pbz) was from Smith Kline & French s.p.a. (Milano, Italy). Final concentration of MEN 10,449 higher than 10 μM could not be tested because of precipitation in the organ bath. Stock solutions of Pbz (100 mM) were made by dissolving the drug in 0.05 N HCl. Water dilutions were made from frozen stock solutions just before use.

Results

Effect of MDL 28, 564

In the RPA, MDL 28,564 acted as a full agonist, being about 17 and 30 times less potent than NKA or NKA (4–10), respectively (Table 2; Figure 1a); its maximal response averaged 91% (95% C.L. = 82–100) of that to NKA. As shown in Figure 1b, the response to MDL 28,564 was competitively antagonized by MEN 10,207 and R 396 with a pA_2 of 7.87 ± 0.03 (n = 6) and 5.49 ± 0.05 (n = 6) respectively. These pA_2 values were similar to those obtained in comparable experimental conditions against NKA, or the selective NK_2 receptor agonist [βAla^8]-NKA (4–10) (Maggi *et al.*, 1990).

The dissociation constant (K_A) as well as the receptor reserve for MDL 28,564 in the RPA was calculated following partial irreversible alkylation with Pbz (Figure 2; Table 3). Both the K_A value (646.5 nM) and receptor reserve found for MDL 28,564 were lower than those for NKA in this organ (Table 3).

In the HT, MDL 28,564 (3–30 μM) beside producing a slight, not concentration-dependent contraction (not exceeding 10% of the response to NKA, or NKA (4–10), Figure 1c, n = 14) competitively antagonized the response to NKA (Figure 1d) or [βAla^8]-NKA (4–10) with pA_2 values of 6.21 ± 0.09 and 6.25 ± 0.12 (n = 12 and 4, respectively). Therefore, in spite of the different mode of action of MDL 28,564, no difference was

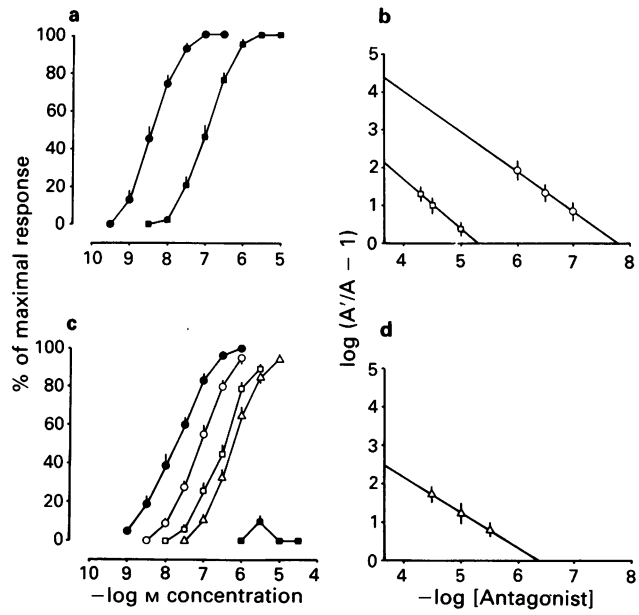

Figure 1 (a) Concentration-response curve to neurokinin A (NKA) (●) or MDL 28,564 (■) in the endothelium-deprived rabbit pulmonary artery (RPA). Each value is mean of 8–12 experiments with s.e. shown by vertical lines. (b) Schild plots showing the antagonism of MDL 28,564-induced contractions of the RPA by MEN 10,207 (○) and R 396 (□). Each value is mean of 4 experiments; s.e. shown by vertical lines. (c) Concentration-response curve to neurokinin A in the hamster isolated trachea (HT) in control (●, n = 12) or in the presence of various concentrations of MDL 28,564 (○, 3 μM; □, 10 μM; △, 30 μM; contact time 15 min for each concentration, n = 4 each); (■) effect of MDL 28,564 alone. Each value is mean of 4–6 experiments; s.e. shown by vertical lines. (d) Schild plot showing the antagonism of the contractile response to NKA in the HT by MDL 28,564. Each value is mean of 4 experiments; vertical lines show s.e.

Table 2 Comparison of the activity of various NK_2 receptor ligands in the endothelium-denuded rabbit pulmonary artery (RPA) and in the hamster trachea (HT)

	RPA			*HT*		
Peptide	pD_2	α	pA_2	pD_2	α	pA_2
NKA	8.10** (8.0–8.20)	1	...	7.30 (7.21–7.39)	1	...
NKA (4–10)	8.34** (8.14–8.54)	1 (0.9–1.1)	...	7.45 (7.30–7.60)	1 (0.9–1.1)	...
MDL 28,564	6.86 (6.74–6.98)	0.91 (0.82–1.0)	...	...	...	6.21 (5.99–6.42)
MEN 10,295	6.31 (6.0–6.61)	0.98 (0.9–1.1)	...	5.71 (5.4–6.01)	0.57 (0.47–0.67)	...
MEN 10,296	6.0 (5.77–6.23)	0.85 (0.77–0.93)	...	5.76 (5.38–6.14)	0.75 (0.65–0.85)	...
MEN 10,207§	...	...	7.89** (7.71–8.06)	...	...	5.94 (5.72–6.18)
MEN 10,282	...	...	6.47 (6.13–6.81)	...	...	6.12 (5.77–6.47)
MEN 10,449	...	...	5.60 (5.33–5.88)	...	...	5.58 (5.25–5.90)
L 659,877	...	...	6.72 (6.50–7.0)	...	...	7.92 (7.84–8.0)

Each value is mean of at least 6 determinations. In parentheses are 95% C.L.
$pD_2 = -\log EC_{50}$.
pA_2 values were obtained by means of the constrained plot method as described by Tallarida *et al.* (1979).
α = intrinsic activity, expressed as a fraction of the maximal response to neurokinin A.
** Significantly different from the corresponding value in HT: $P < 0.01$.
§ Data for MEN 10,207 are from Maggi *et al.*, 1990.

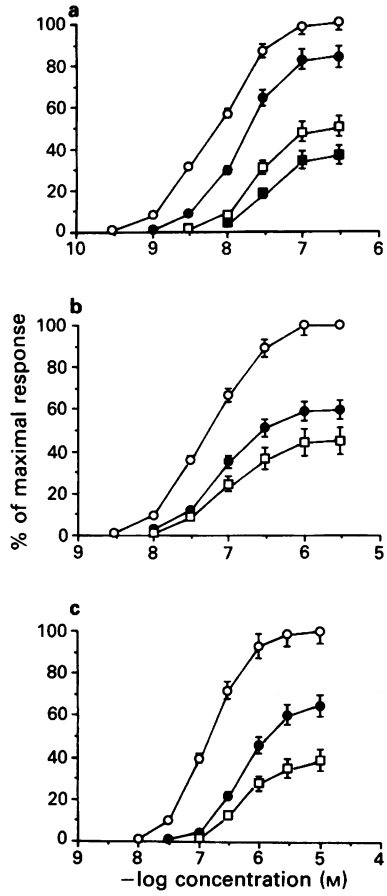

Figure 2 (a) Concentration-response curves to neurokinin A in the endothelium-deprived rabbit pulmonary artery (RPA) before (○) or after 30 min incubation with phenoxybenzamine 10 μM (●), 30 μM (□) and 50 μM (■). (b) Concentration-response curves to neurokinin A in the hamster isolated trachea (HT) before (○) or after 30 min incubation with phenoxybenzamine 10 μM (●) and 20 μM (□). (c) Concentration-response curves to MDL 28,564 in the RPA before (○) or after 30 min incubation with phenoxybenzamine 10 μM (●) and 15 μM (□).

Table 3 Irreversible antagonism by phenoxybenzamine of neurokinin A (NKA) and MDL 28,564 contractile effects in the endothelium-denuded rabbit pulmonary artery (RPA) and of NKA contractile effect in the hamster trachea (HT)

Values	*RPA*		*HT*
Agonists	*MDL 28,564*	*NKA*	*NKA*
EC_{50} (nM)	162.4	8.0**	50.9
	(121.6–203.2)	(6.4–9.6)	(40.9–60.9)
K_A (nM)	645.5	79.8**	169.7
	(344.2–948.8)	(53.0 – 106.5)	(132.1–207.3)
Reserve	3.1	11.1**	3.62
	(2.04–4.16)	(7.4–14.8)	(2.7–4.5)
q (%)	30.3	21.9	33.7
	(22.3–38.2)	(16.1–27.7)	(27.8–39.6)
ρ (%)	26.9	11.6**	24.1
	(20.2–33.5)	(7.9–15.3)	(18.7–29.5)

Each value is mean of at least 12 determinations plus 95% C.L. Phenoxybenzamine (10–50 μM) was left in contact with tissues for 30 min.
EC_{50} = agonist concentration producing 50% of maximal response.
K_A = agonist concentration producing 50% of maximal receptor occupancy.
Reserve = K_A/EC_{50}.
q = fraction of receptors remained unblocked after incubation with phenoxybenzamine.
ρ = fractional occupancy of receptors by NKA or MDL 28,564 required to elicit half maximal response.
** Significantly different from the corresponding value in HT: $P < 0.01$.

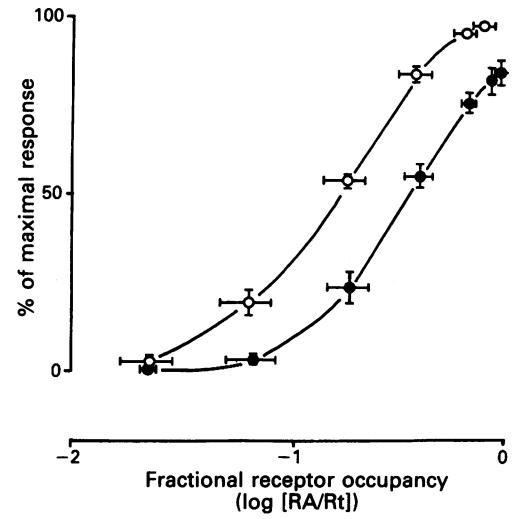

Figure 3 Comparison of the efficacy of neurokinin A (NKA, ○) (ε_{NKA}) relative to that of MDL 28,564 (●) (ε_{MDL}) on the NK_2 receptor of the rabbit pulmonary artery ($\varepsilon_{NKA}/\varepsilon_{MDL}$ = 2.2). Ordinates: percentage of the maximal response to NKA in each experiment. Abscissae: logarithms of the receptor occupancy of various concentration of agonists. Each value represents mean of 6 determinations; s.e. shown by bars.

found between the affinity of this peptide for the NK_2 receptor in the RPA (K_A = 646.5 nM; pK_A = 6.19) or in the HT ($pA_2 = pK_B$ = 6.21 or 6.25).

Effect of other NKA-(4–10) analogues and L 659,877

MEN 10,295 and MEN 10,296, two analogues of [βAla^8]-NKA (4–10) (see Table 1), behaved as full or virtually full agonists in the RPA (Table 2), being 60 and 125 times less potent than NKA, respectively. In the HT they still behaved as agonists, even if with lower potency and intrinsic activity, as compared to the RPA (Table 2).

MEN 10,282 and MEN 10,449, two close analogues of MEN 10,207 (Table 1), acted as competitive antagonists, producing a concentration-dependent rightward shift of the curve to NKA in the RPA. Their affinity values were between 1.5 and 2.5 log units lower than those found for MEN 10,207, in the same organ (Table 2). In the HT, both MEN 10,282 and MEN 10,449 competitively antagonized the contractile effect to NKA, with potencies similar to those observed in the RPA (Table 2). Therefore both compounds were equipotent in the two preparations examined.

L 659,877, a cyclic TK antagonist (McKnight *et al.*, 1988), acted as a potent competitive antagonist of NKA contractile effects, both in RPA and HT (Table 2). In the latter organ L 659,877 was about 15 times more potent than in the RPA.

Receptor reserve for neurokinin A in rabbit pulmonary artery and hamster trachea

The dissociation constants (K_A) calculated for NKA in the RPA and HT by the method of partial irreversible alkylation of NK_2 receptors with Pbz (Figure 2), were found to be close but significantly different from each other (Table 3). The receptor reserve for NKA in the RPA was sizeable, being about 3 times higher than in the HT (Table 3). The receptor occupancy required to obtain half maximal response to NKA averaged 11.6% in the RPA and 24.1% in the HT.

Relative efficacy of neurokinin A and MDL 28,564 in the rabbit pulmonary artery

The relative efficacy of NKA and MDL 28,564 for the NK_2 receptor in the RPA was evaluated by the procedure of Furchgott & Bursztyn (1967) (see Methods for details). By using the K_A values for both NKA and MDL 28,564 in the

RPA, an occupancy-response curve was constructed (Figure 3) from which the relative efficacy between NKA and MDL 28,564 ($\varepsilon_{NKA}/\varepsilon_{MDL}$) was calculated. NKA intrinsic efficacy was 2.2 times higher than that of MDL 28,564 for the NK_2 receptor in the RPA. Such a comparison was impossible in the HT, because of the lack of intrinsic efficacy for MDL 28,564 which is unable to act as agonist up to 30 μM, a concentration sufficient to occupy 98% of total NK_2 receptors.

Discussion

We have previously provided evidence for the existence of NK_2 receptor subtypes in the RPA and HT, which are recognized with very different affinity by certain antagonists, such as R 396 (100 times more potent in the HT than in the RPA) or MEN 10,207 (100 times more potent in the RPA than in the HT) (Maggi *et al.*, 1990). The results of the present investigation point to differences in the character of NK_2 receptors mediating contractility in the RPA and HT, hence they support the concept of NK_2 receptor heterogeneity.

One of the tools employed in this study, the selective NK_2 ligand MDL 28,564 (Buck *et al.*, 1990), showed full agonist activity in the RPA, while it acted as a competitive antagonist in the HT. The contractile responses elicited by MDL 28,564 in the RPA appear to involve the same receptor activated by NKA, as indicated by the similar pA_2 values obtained with MEN 10,207 and R 396 against MDL 28,564 (present findings) or NKA and [βAla^8]-NKA (4–10) (Maggi *et al.*, 1990) as agonists. The behaviour of MDL 28,564 could be explained either in terms of differences in receptor coupling and/or density (tissue factors) between the RPA and HT or by differences existing between the two NK_2 receptors present in the above tissues. The aim of the present work was to verify these possibilities. In order to obtain a measure of the extent of tissue factors in the HT and RPA, we compared the receptor reserve for NKA in these latter organs. Indeed the size of the receptor reserve for NKA observed in the RPA and HT was slightly greater (about 3 times) in the former than that in the latter tissue. Such a difference could explain the lower potency (from 3 to 7 times) observed for NKA and other NK_2 receptor agonists in the HT as compared to the RPA (cf. Maggi *et al.*, 1990 and present findings). However, it appears doubtful that the smaller receptor reserve, measured in the HT, could explain the total loss of agonist activity for MDL 28,564 in this tissue. On the other hand the present results do indicate that the main cause of the behaviour of MDL 28,564 is the different intrinsic efficacy displayed by the compound for the two NK_2 receptors. In fact the intrinsic efficacy of MDL 28,564 in the RPA was only 2.2 times lower than that of NKA, while it was virtually undetectable in the HT. Indirect evidence supporting this hypothesis comes from the results obtained with MEN 10,295 and MEN 10,296, two [βAla^8]-NKA (4–10) analogues showing weaker agonist properties than MDL 28,564 in the RPA. If 'tissue factors', i.e. receptor coupling and density, which are regarded as completely drug-independent parameters, were the determinants for the loss of MDL 28,564 agonist activity in the HT, then these factors would have similarly affected the agonist activity of MEN 10,295 and MEN 10,296 in the former organ. On the contrary MEN 10,295 and 10,296 maintain agonist properties in the HT, demonstrating that tissue factors are of little relevance for the expression of agonist activity in the RPA and HT within this class of peptides. Measurements of affinity constants (true affinity, K_A) for MDL 28,564 and NKA did not provide a means of distinguishing between subtypes. In fact MDL 28,564 binds to both subtypes with comparable affinity (in terms of pK_A or pK_B), and the difference in the K_A value noted for the natural ligand NKA, though significant, is too small to indicate receptor heterogeneity. A similar behaviour of MDL 28,564 has been reported by Buck *et al.* (1990) for other tissues containing NK_2 receptors: the guinea-pig trachea on one hand and the hamster urinary bladder and rat vas deferens on the other (see also Introduction). These findings suggest that the pseudopeptide MDL 28,564, derived from the NKA (4–10) sequence by reduction of the bond between positions 9 and 10, recognizes putative NK_2 receptor subtypes with similar affinity but possesses sufficient intrinsic efficacy for only one of the subtypes. On the basis of these data one may speculate that the terminal region of the sequence (e.g. the residues 9 and 10) is crucially involved in receptor activation, while the remaining sequence contributes to receptor recognition and binding.

An interesting finding emerges from the comparison of various antagonists containing D-Trp in their sequence. For example MEN 10,282 and MEN 10,449, two close analogues of MEN 10,207, are as potent as the parent compound in the HT but are weaker antagonists than MEN 10,207 in the RPA. This observed reduction of antagonist potency occurring in the latter tissue, suggests that MEN 10,207 possesses rather exacting structural requirements which determine its ability to bind with high affinity to the RPA NK_2 subtype. On the other hand the cyclic antagonist L 659,877 exhibits greater potency (15 times) in inhibiting NK_2 receptor-mediated responses in the HT relative to the RPA, suggesting that, among antagonists, the ability to selectively recognize subtypes is not confined to the NKA sequence. From the limited examples presented here it appears that the NK_2 receptor of the HT may accommodate a wider range of chemical moieties.

In conclusion this investigation provides further evidence for NK_2 receptor heterogeneity. On the basis of the present and previous findings (Maggi *et al.*, 1990) we conclude that the NK_2 receptor subtype present in the RPA is recognized with the highest affinity by MEN 10,207 and with high intrinsic efficacy (full agonist activity) by MDL 28,564. On the contrary the NK_2 receptor subtype present in the HT is recognized with the highest affinity by L 659,877, and with low (or absent) intrinsic efficacy (antagonist activity) by MDL 28,564.

References

ARUNLAKSHANA, O. & SCHILD, H.O. (1959). Some quantitative uses of drug antagonists. *Br. J. Pharmacol. Chemother.*, **14**, 48–58.

BUCK, S.H. & BURCHER, E. (1987). Differential sensitivity to phenoxybenzamine alkylation among types of neurokinin binding sites. *Neuropeptides*, **9**, 33–39.

BUCK, S.H., BURCHER, E., SCHULTZ, C.W., LOVENBERG, W. & O'DONAHUE, T.L. (1984). Novel pharmacology of substance K binding sites: a third type of tachykinin receptor. *Science*, **226**, 987–989.

BUCK, S.H., HARBESON, S.L., HASSMANN III, C.F., SHATZER, S.A., ROUISSI, N., NANTEL, F. & VAN GIERSBERGEN, P.L.M. (1990). [Leu$^9\psi$(CH$_2$NH)Leu^{10}]-Neurokinin A (4–10) (MDL 28,564) distinguishes tissue tachykinin peptide NK_2 receptors. *Life Sci.*, **47**, PL-37-PL41.

BUCK, S.H., PRUSS, R.M., KRSTENANSKY, J.L., ROBINSON, P.J. & STAUDERMAN, K.A. (1988). A tachykinin peptide receptor joins an elite club. *Trends Pharmacol. Sci.*, **9**, 3–5.

DION, S., D'ORLEANS-JUSTE, P., DRAPEAU, G., RHALEB, N.E., ROUISSI, N.E., TOUSIGNANT, C. & REGOLI, D. (1987). Characterization of neurokinin receptors in various isolated organs by the use of selective agonists. *Life Sci.*, **41**, 2269–2276.

D'ORLEANS-JUSTE, P., DION, S., DRAPEAU, G. & REGOLI, D. (1985). Different receptors are involved in the endothelium-mediated relaxation and the smooth muscle contraction of the rabbit pulmonary artery in response to substance P and related neurokinins. *Eur. J. Pharmacol.*, **125**, 37–44.

DRAPEAU, G., D'ORLEANS-JUSTE, P., DION, S., RHALEB, N.E., ROUISSI, N.E. & REGOLI, D. (1987). Selective agonists for substance P and neurokinin receptors. *Neuropeptides*, **10**, 43–48.

FURCHGOTT, R.F. (1966). The use of β-haloalkylamines in the differentiation of receptors and in the determination of dissociation constants of receptor-agonist complexes. In *Advances in Drug Research*. ed. Harper, N.J. & Simmonds, A.B. Vol. 3, pp. 21–55. New York, N.Y.: Academic Press, Inc.

FURCHGOTT, R.F. & BURSZTYN, P. (1967). Comparison of dissociation constants and of relative efficacies of selected agonists acting on parasympathetic receptors. *Ann. N.Y. Acad. Sci.*, **144**, 882–899.

HARBESON, S.L., BUCK, S.H., HASSMANN III, C.F. & SHATZER, S.A. (1990). Synthesis and biological activity of [$\psi(CH_2NH)$] analogs of neurokinin A (4–10). In *Peptides: Chemistry, Structure and Biology*. ed. Rivier, J.E. & Marshall, G.R. pp. 180–181. Leiden: Escom.

HERSHEY, A.D. & KRAUSE, J.E. (1990). Molecular characterization of a functional cDNA encoding the rat substance P receptor. *Science*, **247**, 958–962.

KENAKIN, T.P. (1987). *Pharmacologic Analysis of Drug-Receptor Interaction*. New York, N.Y.: Raven Press.

LEE, C.M., CAMPBELL, N.J., WILLIAMS, B.J. & IVERSEN, L.L. (1986). Multiple tachykinin binding sites in peripheral tissue and brain. *Eur. J. Pharmacol.*, **130**, 209–216.

LIN, C.W. & MUSACCHIO, J.M. (1983) The determination of dissociation constants for substance P and substance P analogues in the guinea-pig ileum by pharmacological procedures. *Mol. Pharmacol.*, **23**, 558–562.

MAGGI, C.A., GIULIANI, S., SANTICIOLI, P., REGOLI, D. & MELI, A. (1987). Peripheral effects of neurokinins: functional evidence for the existence of multiple receptors. *J. Autonom. Pharmacol.*, **7**, 11–21.

MAGGI, C.A., PATACCHINI, R., GIULIANI, S., ROVERO, P., DION, S., REGOLI, D., GIACHETTI, A. & MELI, A. (1990). Competitive antagonists discriminate between NK_2 tachykinin receptor subtypes. *Br. J. Pharmacol.*, **100**, 588–592.

MAGGI, C.A., PATACCHINI, R., ROVERO, P. & MELI, A. (1989). The hamster isolated trachea: a new preparation for studying NK-2 receptors. *Eur. J. Pharmacol.*, **166**, 435–440.

MASU, Y., NAKAYAMA, K., TAMAKI, H., HARADA, Y., KUNO, M. & NAKANISHI, S. (1987). cDNA cloning of bovine substance-K receptor through oocyte expression system. *Nature*, **329**, 836–838.

McKNIGHT, A.T., MAGUIRE, J.J., WILLIAMS, B.J., FOSTER, A.C., TRIDGETT, R. & IVERSEN, L.L. (1988). Pharmacological specificity of synthetic peptides as antagonists at tachykinin receptors. *Reg. Pept.*, **22**, 127.

REGOLI, D., DRAPEAU, G., DION, S. & COUTURE, R. (1988). New selective agonists for neurokinin receptors: pharmacological tools for receptor characterization. *Trends Pharmacol. Sci.*, **9**, 290–295.

REGOLI, D., DRAPEAU, G., DION, S. & D'ORLEANS-JUSTE, P. (1987). Pharmacological receptors for substance P and neurokinins. *Life Sci.*, **40**, 109–116.

REGOLI, D., DRAPEAU, G., DION, S. & D'ORLEANS-JUSTE, P. (1989). Receptors for substance P and related neurokinins. *Pharmacology*, **38**, 1–15.

ROVERO, P., PESTELLINI, V., PATACCHINI, R., GIULIANI, S., SANTICIOLI, P., MAGGI, C.A., MELI, A. & GIACHETTI, A. (1989). A potent and selective agonist for NK_2 tachykinin receptor. *Peptides*, **10**, 593–595.

ROVERO, P., PESTELLINI, V., MAGGI, C.A., PATACCHINI, R., REGOLI, D. & GIACHETTI, A. (1990). A highly selective NK_2 tachykinin receptor antagonist containing D-tryptophan. *Eur. J. Pharmacol.*, **175**, 113–115.

TALLARIDA, R.J., COWAN, A. & ADLER, M.W. (1979). pA_2 and receptor differentiation: a statistical analysis of competitive antagonism. *Life Sci.*, **25**, 637–654.

VAN GIERSBERGEN, P.L.M., SHATZER, S.A., HENDERSON, A.K., LAI, J., NAKANISHI, S., YAMAMURA, H.I. & BUCK, S.H. (1991). Characterization of a tachykinin peptide NK_2 receptor transfected into murine fibroblast B82 cells. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 1661–1666.

VAUGHT, J.L., SCOTT, R.W. & JACOBY, H.I. (1986). A differentiation between peripheral and central neurokinin receptors using phenoxybenzamine. *Eur. J. Pharmacol.*, **125**, 325–331.

YOKOTA, Y., SASAI, Y., TANAKA, K., FUJIWARA, T., TSUCHIDA, K., SHIGEMOTO, R., KAKIZUKA, A., OHKUBO, H. & NAKANISHI, S. (1989). Molecular characterization of a functional cDNA for rat substance P receptor. *J. Biol. Chem.*, **264**, 17649–17652.

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